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Selective modulation of the prostaglandin F2 α pathway markedly impacts on endometriosis progression in a xenograft mouse model

Ahmad, Syed Furquan^{1,2*}, Akoum, Ali^{1,†} and Horne, Andrew W²

¹Laboratoire d'Endocrinologie de la Reproduction, Centre de recherche, CHU de Québec-HSFA, Faculté de Médecine, Université Laval, Québec, Canada

²MRC Centre for Reproductive Health, The University of Edinburgh, Queen's Medical Research Institute, Edinburgh EH16 4TJ, United Kingdom

*Corresponding author and person to whom reprint requests should be addressed: Dr. Syed Furquan Ahmad, MRC Centre for Reproductive Health, QMRI, 47 Little France Crescent, Edinburgh EH16 4TJ. E-mail: furquan.ahmad@ed.ac.uk

[†]Deceased

Abstract

Study hypothesis: Selective activation or blockade of the prostaglandin (PG) F2 α receptor (FP receptor) affects ectopic endometrial tissue growth and endometriosis development.

Study finding: FP receptor antagonists might represent a promising approach for the treatment of peritoneal endometriosis.

What is known already: Eutopic and ectopic endometrium from women with endometriosis exhibit higher expression of key enzymes involved in the PGF2 α biosynthetic pathway. It has also been shown that the PGF2 α -FP receptor interaction induces angiogenesis in human endometrial adenocarcinoma.

Study design, samples/materials, methods: For this study, a mouse model of endometriosis was developed by inoculating human endometrial biopsies into the peritoneal cavity of nude mouse (n=15). Mice were treated with AL8810 (FP receptor antagonist), fluprostenol (FP receptor agonist) or PBS. Endometriosis like lesions were collected and analysed for set of markers for angiogenesis, tissue remodeling, apoptosis, cell proliferation and capillary formation using qPCR and immunohistochemistry.

Main results and the role of chance: We found that selective inhibition of the FP receptor with a specific antagonist, AL8810, led to a significant decline in the number ($p < 0.01$) and size of endometriosis-like lesions ($p < 0.001$), down-regulated the expression of key mediators of tissue remodelling (MMP9, $p < 0.05$) and angiogenesis (VEGF, $p < 0.01$) and upregulated the pro-apoptotic factor (Bax, $p < 0.01$) as compared to controls. Immunohistochemical analyses further showed a marked decrease in cell proliferation and capillary formation in endometrial implants from AL8810 treated mice, as determined by proliferating cell nuclear antigen (PCNA) and von

Willebrand factor (vWF) immunostaining, respectively. Moreover, Fluperostenol, a selective FP receptor agonist, showed the opposite effects.

Limitations, reasons for caution: We carried out this study in nude mice, which have low levels of endogenous estrogens which may affect the lesion growth. Caution is required when interpreting these results to women.

Wider implications of the findings: This study extends the role of PG signaling in endometriosis pathogenesis and points towards the possible relevance of selective FP receptor antagonism as a targeted treatment for endometriosis.

Large scale data: N/A

Study funding and competing interest(s): This work was supported by grant MOP-123259 to the late Dr Ali Akoum from the Canadian Institutes for Health Research. The authors have no conflict of interest.

Keywords: Endometriosis, Prostaglandins, PGF2 α , FP receptor, AL8810, Fluprsostenol

Introduction

Endometriosis is a chronic inflammatory disease affecting 6-10% of reproductive-age women. It is characterized by the presence of functional endometrial tissue outside the uterine cavity and is associated with pelvic pain, dysmenorrhea and infertility (Giudice, 2010, Macer and Taylor, 2012). The most accepted explanation of the extra-uterine localization of endometrial tissue is mainly based on the common occurrence of retrograde menstruation, where menstrual endometrial tissue is disseminated into the peritoneal cavity via the Fallopian tubes and is capable of implanting and developing into endometriosis lesions (Sampson, 1927). Although the full range of mechanisms responsible for the development of endometriosis lesions remain to be clarified, a number of recent GWAS studies have highlighted genetic risk factors that may contribute to life-time risk (Rahmioglu, et al., 2014).

Prostaglandins (PGs) are well-known regulators of signalling within the female reproductive tract and their roles in ovarian function, embryo implantation and menstruation are well described (Sales and Jabbour, 2003, Vilella, et al., 2013). PGs have also been implicated in endometrial pathologies such as endometriosis and endometrial cancer (Jabbour and Sales, 2004). In the female reproductive tract, the E and F series of prostanoids are synthesized from arachidonic acid via a series of oxidation steps involving cyclooxygenase (COX-1, -2) enzymes and the PG E and F synthases, respectively (Narumiya and FitzGerald, 2001). Notably the aldo-ketoreductases AKR-1C3 and AKR-1B1, which have PGF synthase activities, have also been localised to the human endometrium (Fortier, et al., 2008). After biosynthesis, PGF₂ α is transported out of the cell by means of a carrier-mediated process where it exerts

autocrine/paracrine functions through a G protein receptor (GPCR)-mediated interaction (Chan, et al., 1998). The GPCR that binds human $\text{PGF2}\alpha$, the FP receptor, has been cloned, and its activation leads to coupling of the G protein Gq, activation of phospholipase C (PLC) and release of inositol trisphosphate (IP3) and diacylglycerol (Abramovitz, et al., 1994).

Endometriosis is a neuroinflammatory disorder associated with pain and infertility. It has been suggested that altered endometrial functions contribute both to the aetiology of the disorder and development of infertility (Macer and Taylor, 2012, Taylor, et al., 1999). Notably ectopic, extra-uterine endometrial tissue found in lesions retain certain hallmarks of eutopic endometrium including a dependence on oestrogens for continued growth (Hudelist, et al., 2007). This observation has led to the adoption of hormonal suppression as a widely used medical therapy, however this can result in development of unacceptable side effects including a pseudo-menopause (due to a hypo-oestrogenic environment) or pseudo-pregnancy (due to a progestin-dominant environment) (Bulun, 2009). Although hormonal manipulations are often used as first-line therapy as well as after surgery to prevent recurrence of symptoms, their long term use is associated with loss of bone density, low mood and increased risk for uterine and ovarian cancers (Swiersz, 2002, Vercellini, et al., 2014). Recurrence rates are 50%-60% within a year after cessation of hormone therapy (Guo and Olive, 2007, Kyama, et al., 2008) and there is therefore an urgent need to identify non-steroidal therapeutic targets for the treatment of endometriosis.

Our recent findings suggested a significant deregulation of $\text{PGF2}\alpha$ biosynthesis and action in women with endometriosis at multiple levels (Rakhila, et al., 2013). These included an over-expression of COX2, the inducible rate limiting enzyme in PG synthesis, in eutopic and ectopic endometrium of women with endometriosis and an up-regulation of AKR-1C1 in endometriosis lesions (Rakhila, Carli, Daris, Lemyre, Leboeuf and Akoum, 2013). In addition, $\text{PGF2}\alpha$ -FP

receptor interaction has recently been shown to induce angiogenesis in human endometrial adenocarcinoma (Sales, et al., 2005). The present study was therefore designed to investigate, using a heterologous mouse model of endometriosis, the impact of treatment with selective FP receptor modulators on ectopic endometrial tissue growth and endometriosis development. Our data suggest that treatment with FP receptor antagonists might represent a promising approach for the treatment of peritoneal endometriosis.

Materials and Methods

Human tissue resource

Endometrial biopsies (n=3/patient) were obtained from five patients undergoing surgical explorative laparoscopy or hysterectomy for benign conditions (confirmed as not having endometriosis and not receiving anti-inflammatory or hormonal medication for at least three months before surgery). These patients signed an informed consent for a research protocol approved by Saint-François d'Assise Hospital ethics committee on human research (Laval University, Québec, Canada).

Animal handling and treatment

For this study, 15 six to eight week-old female athymic Nude-Foxn1nu mice (Harlan Laboratories, Indianapolis, IN, USA) were used. The protocol was approved by the committee of animal protection of Laval University and in-vivo experiments were performed according to the Canadian committee of animal's protection (CPA) rules. Mice were housed under laminar-flow filtered hoods in rooms maintained at 28°C with a 12:12-hour light-dark cycle. Housing

materials, food, and water were sterilized before use. A schematic illustration of the experimental design is shown in Figure 1a. Human endometrial tissue samples collected were placed in cold sterile PBS and dissected into small pieces ($\sim 1\text{mm}^3$) and labelled with 8×10^{-6} M carboxyfluorescein diacetate, succinimidyl ester (CFDA-SE, Invitrogen, Burlington, ON, Canada) diluted in PBS for 20 min at room temperature. Tissue fragments were washed twice in PBS and labelling was confirmed by fluorescence stereomicroscopy (Carl Zeis, Germany) equipped with a fluorescein isothiocyanate (FITC) filter to detect the fluorescence of CFDA SE at ex465/em535.

For induction of endometriosis mice, were given buprenorphine (1.68 g per mouse) by intradermal injection for analgesia, then anesthetized with a mixture of oxygen (1.5: 1) and isoflurane (3% to 4%) (Abbot Laboratories, Saint-Laurent, Quebec, Canada). A small (1 cm) cutaneous and peritoneal incision was made in a sterile environment, and 0.1 mL of PBS containing 13 CFDA-SE labelled endometrial tissue fragments were injected into the peritoneal cavity using a micropipette (Essentially, biopsy from 1 patient was chopped into 39 fragments). The incision was closed with Coated NB (polyglactin 910) sutures (Ethicon Johnson & Johnson, Markham, ON, Canada) for the peritoneal tissue and MikRon autoclip 9 mm (Clay Adam Brand, Sparks, MD, USA) for the cutaneous tissue. The mice did not receive any exogenous supply of estrogen and they were monitored daily for comfort, survival, and weight for 12 days after initial surgery. To manipulate FP receptors, the mice were treated with the FP antagonist AL8810 [(5Z,13E)-(9S,11S,15R)-9,15-dihydroxy-11-fluoro-15-(2-indanyl)-16,17,18,19,20-pentanoic acid] (Cayman Chemical Company, Ann Arbor, MI, USA) (Griffin, et al., 1999) or the FP receptor agonist Fluprostenol (Cayman Chemical Company) (Jin, et al., 2006). On day 12 mice were injected intra-peritoneally with AL8810 (5 mg/kg), Fluprostenol (0.15 mg/kg)

(Glushakov, et al., 2013, Jin, Lu, Paoni and Yang, 2006) or PBS as a vehicle control. Additional daily injections were given on days 13-18 with daily monitoring of weight. On day 19 (Fig 1a) animals were anesthetized and then euthanized in an atmosphere saturated by CO₂. The abdominal cavity was examined under a fluorescence stereomicroscope using Axiocam camera and Axiovisio Rel 4.8 software (Carl Zeis, Germany). The number of lesions/mouse was recorded and they were measured, and photographed before being recovered and processed for RNA or histology as detailed below. The area of lesion was measured using ImageJ software by multiplying the maximum and minimum diameter of the lesion.

RNA extraction and qRT-PCR

Endometriosis lesions were dissected under fluorescence stereomicroscopy from the surrounding tissue and RNA was extracted with Trizol reagent (Invitrogen) according to the manufacturer's instructions. The total RNA concentration was measured by using a NanoDrop spectrophotometer and then RNA was reverse transcribed using random hexamers. qRT-PCR was performed using an ABI 7000 Thermal Cycler (Applied Biosystems, Foster City, CA). Each PCR reaction contained 2 µL of reverse transcriptase product, 0.5 µL of primer (final concentration, 0.1 mM), 12.5 µL of SYBR Green PCR Master Mix (Invitrogen) containing TaqDNA polymerase buffer, deoxynucleotide triphosphate mix, SYBR green I, MgCl₂, and TaqDNA polymerase. Primers were designed with Primer Premier 5 software to cross intron-exon boundaries, and specificity to human tissue was verified with Basic Local Alignment Search Tool (BLAST) (Table 1). Samples were tested in duplicate, and, for each reaction, negative controls without RNA or reverse transcriptase, RNA from mouse tissue (negative control) and RNA from endometrial tissue (positive control) were added.

Histology and Immunohistochemistry

Lesions were removed carefully and fixed in 10% formalin and then embedded into paraffin. Cryosections (5 μ m) of paraffin embedded tissue sections were rehydrated and stained with hematoxylin and eosin. For immunostaining, endometriotic lesions were mounted on poly-L-lysine-coated microscope glass slides and immunostained as described previously (Rakhila, Carli, Daris, Lemyre, Leboeuf and Akoum, 2013). The primary antibodies used were anti-vWF (Dako, Burlington, ON, Canada) (A0082, 1:100) and anti-PCNA (Dallas, TX, USA) (sc-25280, 1:100). Tissue sections incubated without the primary antibody were included as negative controls. Secondary antibodies used were HRP-conjugated goat anti-mouse IgG Jackson (115-035-146) (1:2,000 dilution in PBS/BSA/Tween) for PCNA and a biotin-conjugated goat anti-rabbit IgG (E 0432, Dako) (1:2,000 dilution in PBS/BSA/Tween) for vWF. Microphotographs were captured using the Image Pro Express program (Meyer Instrument, Houston, TX, USA).

Statistical Analysis

Data related to the number and volume of lesions followed a nonparametric distribution and were analysed using Mann-Whitney *U* test. Data related to the weight of mice and qRT-PCR followed a Gaussian distribution and were analysed using ANOVA and Bonferroni test (GraphPad Software, San Diego, CA). Differences were considered as statistically significant using $p < 0.05$.

Results

AL8810 and Fluprostenol treatments engender opposite effects on ectopic endometrial tissue growth

The animals exposed to either AL8810 or Fluprostenol showed no signs of any discomfort or weight loss. The engraftment rate was similar in all control mice (5-6 implants survived out of 13 fragments) and was not patient dependent. At the time of lesion recovery endometriotic-like implants were found scattered throughout the abdominal cavity of the mice. Initial examination revealed that lesions were smaller in AL8810-treated mice compared with those in fluprostenol- and vehicle-treated mice (Figure 1B). Histological evaluation of harvested lesions showed endometrial tissue composed of epithelial glands and compact stroma (Figure 2A). In mice treated with AL8810, endometriotic tissue implants were small cystic structures with degenerating endometrial glands and scattered stromal cells, whereas in mice treated with Fluprostenol, endometrial tissue closely adhered to the host tissue and exhibited many well-defined, secretory and active endometrial glands and compact stroma. Mice treated with AL8810 developed fewer lesions and the lesions detected were smaller, compared with Fluprostenol treated mice or control mice, which clearly had larger, more and well defined endometriotic lesions. Statistical analyses showed that the mean number and size of endometriotic lesions were significantly decreased in AL8810-treated mice, compared to vehicle-treated control mice ($p < 0.001$ and $p < 0.01$ respectively), but they were significantly increased in Fluprostenol-treated mice ($p < 0.01$ and $p < 0.001$ respectively) (Figure 2B). Endometriosis lesions were found at several sites, including the peritoneum, intestines, peritoneal fat, liver and kidney. However, in AL8810 treated mice lesion development was limited only to the peritoneal fat.

Expression of PGE2 and PGF2 α biosynthetic and catabolic enzymes are altered by AL8810 and Fluprostenol treatments

In lesions recovered from AL8810-treated mice, concentrations of COX2 mRNA were reduced compared to lesions from control mice ($p < 0.05$). Treatment with Fluprostenol significantly increased COX2 mRNA concentrations as compared to vehicle control ($p < 0.01$) (Figure 3A). Concentrations of COX-1 mRNA was not altered by either treatment (Figure 3B). The expression of the PGF2 α biosynthetic enzyme AKR-1C3 was significantly decreased in lesions from AL8810-treated mice ($p < 0.01$), but was significantly increased following Fluprostenol treatment ($p < 0.01$) (Figure 3C). However, the expression of AKR-1B1 did not show any significant changes in response to AL8810 or Fluprostenol (Figure 3D). Analysis of specific PGE2 biosynthetic enzymes showed that mPGES-1 and mPGES-2 were down-regulated in lesions from AL8810-treated mice, but up-regulated in those from Fluprostenol-treated mice as compared to controls ($p < 0.05$, $p < 0.001$) (Figure 3, E-F). Concentrations of 15-PGDH mRNA, the catabolic enzyme of PGE2 and PGF2 α , were significantly up-regulated in lesions from AL8810-treated mice ($p < 0.001$), but significantly down-regulated in lesions from Fluprostenol-treated mice ($p < 0.01$) as compared to controls (Figure 3H).

AL8810 and Fluprostenol modulate the expression of tissue remodelling and angiogenic factors

We next assessed the expression levels of MMP-9, an important tissue-remodelling factor that is up-regulated in active endometriotic lesions in women (Weigel, et al., 2012) and a mediator of PGF2 α signalling pathway (Sales, List, Boddy, Williams, Anderson, Naor and Jabbour, 2005). Treatment of mice with AL8810 showed significantly down-regulated MMP-9 mRNA

concentrations as compared to control ($p < 0.05$). In contrast, Fluprostenol treatment up-regulated MMP-9 compared to AL8810 ($p < 0.01$) (Figure 4A). Our data further showed that AL8810 treatment, up-regulated mRNA concentrations of TIMP-1, a natural tissue inhibitor of MMP-9 (Brew and Nagase, 2010) as compared to vehicle control ($p < 0.01$), whereas Fluprostenol treatment caused a down-regulation of TIMP-1 mRNA levels ($p < 0.01$). (Figure 4B). We next assessed the expression of VEGF, a major angiogenic factor, that is up-regulated in human endometriosis lesions (Donnez, et al., 1998). Data displayed in Figure 4C showed that VEGF mRNA levels were significantly reduced in lesions from mice treated with AL8810 compared to vehicle-treated control mice ($p < 0.01$), but significantly increased in mice treated with Fluprostenol ($p < 0.05$).

AL8810 and Fluprostenol alter the expression of survival/apoptotic factors in endometriotic lesions

As shown in Figure 5, the mRNA expression level of Bax, a pro-apoptotic factor, was up-regulated in endometriosis-like lesions from AL8810-treated mice ($p < 0.01$), but was down-regulated in lesions from Fluprostenol-treated mice ($p < 0.001$) as compared with lesions from control mice treated with vehicle (Figure 5A). Conversely, treatment with AL8810 showed a down-regulation of mRNA levels of the anti-apoptotic factor Bcl-2. while it was up-regulated in mice treated with Fluprostenol compared to AL8810 mice ($p < 0.05$) (Figure 5B).

Immunohistochemical analysis of proliferation and blood capillary formation

Due to the limited number of endometriotic lesions from AL8810-treated mice, it was not possible to test every protein, so we focussed on a few key processes. Immunolocalisation of PCNA, a marker of cell survival and proliferation (Weigel, Kramer, Schem, Weners, Alkatout,

Jonat, Maass and Mundhenke, 2012, Yu, et al., 1991), showed that numbers of PCNA positive cells were decreased in AL8810-treated lesions but increased in Fluprostenol-treated lesions compared with controls (Figure 6). We also immunolocalised vWF (Von Willebrand Factor), an endothelial cell marker, (Zanetta, et al., 2000) and found that the density of microvessels was increased in lesions from Fluprostenol-treated mice, while it was difficult to detect vWF positive cells in lesions from AL8810-treated mice (Figure 7).

Discussion

In our study, we used a heterologous model of endometriosis to investigate the effect of in-vivo manipulation of an FP-selective agonist (AL8810) and an FP-selective antagonist (Fluprostenol) on lesion size and the concentrations of key mRNAs within the human tissue. In control and fluprostenol treated mice, the engraftment of lesions was found to occur throughout the peritoneal cavity attached to the intestine, kidney, liver and peritoneal wall while the lesions found in AL8810 treated mice were mainly found in peritoneal fat. Our data showed that AL8810 resulted in a marked diminution of the size and number of endometriosis-like lesions and significant changes in the mRNA expression of major molecular mediators of angiogenesis, tissue remodelling, apoptosis and PG biosynthesis, as well as inhibitory effects on markers of cell proliferation and development of micro-vessels in endometriotic implants. In contrast, Fluprostenol, had significant but opposite effects on these pathways and instead favoured cell proliferation, angiogenesis and the growth of endometrial implants.

Cumulative evidence supports a significant role for PGs in the pathophysiology of endometriosis. Our previous data showed distinct expression patterns of PG biosynthetic enzymes in ectopic and

ectopic endometrial tissues of women with endometriosis and a marked increase in the expression levels of the rate-limiting COX2 and the specific terminal synthases for PGE2 (mPGES-1, mPGES-2 and cPGES) and PGF2 α (AKR-1C3) in endometriotic lesions (Rakhila, Carli, Daris, Lemyre, Leboeuf and Akoum, 2013). This is consistent with findings from other studies (Matsuzaki, et al., 2004, Ota, et al., 2001, Sun, et al., 2004), supporting an increase in local production of PGs in endometriosis tissue deposits. Elevated levels of PGE2 and PGF2 α are also found in the peritoneal fluid of women with endometriosis (Dawood, et al., 1984). Although well recognised as major mediators of pain and inflammation, PGs have also been shown to exert a wide array of biological functions and to possess direct and indirect growth-promoting, angiogenic and tissue remodelling effects (Ricciotti and FitzGerald, 2011). Based on our evidence and that of other studies, we hypothesized that selective blockade of cell receptivity to PGs may represent an interesting treatment avenue for endometriosis. PGE₂ has four known cognate receptors, namely EP1, EP2 EP3 and EP4, and recent studies have shown that targeting EP2 and EP4 may inhibit the growth and survival of human endometriotic cell in vitro (Lebovic, et al., 2013). PGF2 α has only one known cognate receptor, the F-series-prostanoid (FP). Therefore, it is tempting to speculate that specific inhibition of PGF2 α signalling via its specific receptor is more achievable as a potential therapeutic option.

Extensive cell proliferation, tissue remodelling and angiogenesis and aberrant apoptosis occur at the ectopic sites where endometrial tissue deposits develop into endometriotic lesions. In this study, AL8810 down-regulated the expression of Bcl-2, which would have favoured cell survival, and concomitantly up-regulated the expression of Bax, a key pro-apoptosis regulatory protein (Basu and Haldar, 1998). Meanwhile, the FP agonist Fluprostenol displayed opposite effects both on Bcl-2 and Bax expression. Taken together our data suggests that blocking PGF2 α

would favour cell death and endometriotic lesion regression. The finding of an increased expression of PCNA, a marker of cell proliferation (Yu, Hall, Fletcher, Camplejohn, Waseem, Lane and Levison, 1991), in the Fluprostenol-treated mice and a decreased expression of this marker in AL8800-treated animals is consistent with these data and suggests a plausible impact of FP antagonism on cell proliferation.

Recently, a novel pro-angiogenic role for PGF2 α has been described in endometrial adenocarcinoma (Sales, List, Boddy, Williams, Anderson, Naor and Jabbour, 2005). This PG has been shown to activate inositol-1,4,5-triphosphate in autocrine and paracrine manners and thereby initiate ERK signalling via the activation of MMPs, transphosphorylation of epidermal growth factor receptor (EGFR) and release of VEGF, which promotes angiogenesis by acting on adjacent endothelial cells (Sales, et al., 2004). The involvement of MMPs and VEGF in the growth and neovascularisation of endometriotic lesions is well documented. These molecules show an up-regulated expression locally in endometriotic lesions and peritoneal macrophages as well as in the uterine eutopic endometrial tissue. Their levels are also elevated in the peritoneal fluid of women with endometriosis (Chung, et al., 2002, Collette, et al., 2006, Donnez, Smoes, Gillerot, Casanas-Roux and Nisolle, 1998). Our study showed that specific blockade of PGF2 α signalling using a specific antagonist of its receptor decreased the expression of VEGF and MMP-9 in endometriotic lesions. Beyond its well-known proteolytic activity, MMP-9 is endowed with a variety of biological functions. This gelatinase is involved in extracellular matrix remodelling in the early angiogenic phase of vascular bud and sprout formation (van Hinsbergh and Koolwijk, 2008) and plays an important role in tumourigenesis and tissue invasion (Hua, et al., 2011). MMP-9 shows an increased expression in both ectopic and eutopic endometrial tissues of women with endometriosis, according to our and other previous studies, acts as a potent

mediator of inflammation (Bellehumeur, et al., 2005) and may contribute to endometriosis progression and dissemination (Chung, Lee, Moon, Hur, Park, Wen and Polan, 2002, Collette, Maheux, Mailloux and Akoum, 2006). Interestingly, treatment with AL8810 resulted in a parallel up-regulation of TIMP-1, a natural tissue inhibitor for MMP-9, (Brew and Nagase, 2010) which suggests the induction of a disequilibrium that may promote endometrial tissue invasion and growth within the host peritoneal tissue and the development of new blood vessels. In keeping with these findings, immunohistochemical analyses revealed that AL8810 effectively attenuated angiogenesis in endometriotic lesions, as indicated by a marked reduction in vWF-positive microvessels, and that Fluprostenol stimulated cell proliferation and capillary ingrowth.

In this study, we demonstrated that specific blockage of $\text{PGF2}\alpha$ -FP receptor signalling acted both upstream by inhibiting the expression of the rate-limiting enzyme COX2 and downstream by down-regulating the expression of the specific terminal synthases of $\text{PGF2}\alpha$ (AKR-1C3) and PGE2 (mPGES-1, mPGES-2 and cPGES). Furthermore, this was paralleled by a significant increase in the PG catabolic enzyme 15-PGDH, thereby suggesting a catabolic shift that we propose leads to a diminution of PG levels. Interestingly, selective activation of cell signalling using a specific FP receptor agonist led to opposite effects on the $\text{PGF2}\alpha$ and PGE2 biosynthesis pathways. Our data suggest that $\text{PGF2}\alpha$ -FP receptor signalling influences the biosynthesis of PGE2 and points to a mutual regulatory mechanism between $\text{PGF2}\alpha$ and PGE2. This is consistent with previous cell signalling studies showing reciprocal crosstalk between the FP receptor and PGE2 receptor EP2 in endometrial cells (Abera, et al., 2010, Sales, et al., 2008) and some evidence that $\text{PGF2}\alpha$ and PGE2 can both activate the FP receptor. Our findings further suggest that the $\text{PGF2}\alpha$ -FP receptor signalling promotes ectopic endometrial tissue growth and make plausible the involvement of PGE2 signalling.

One limitation of our model is that the success rate for implant survival was not high. One explanation could be that nude mice have lower levels of endogenous estrogen. It is also important to note that nude mice do not have T or B cells but they do possess NK cells and macrophages (Budzynski and Radzikowski, 1994) that can exhibit a partial immune response to clear foreign tissue.

Most current medical treatments of endometriosis inhibit the pro-proliferative impact of oestrogens on ectopic lesions via suppression of ovarian steroidogenesis using oral contraceptives, aromatase inhibitors or gonadotropin releasing hormone analogues. Although use of COX-2 inhibitors could be beneficial, their clinical application is of concern because of reported cardiovascular and gastro-intestinal side effects (Howes, 2007). Given the promising data obtained using our well validated mouse model, we speculate that selective inhibition of the action of PGF2 α may represent an alternative targeted treatment for endometriosis.

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Authors' Contribution

AA and SFA designed the study. SFA carried out the experiments and generated the data. AA supervised the experiments and data analysis and reviewed the first draft of the manuscript. SFA and AH wrote the final manuscript.

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Conflict of Interest

The authors declare they have no conflicts of interest.

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Figure Legends

Figure 1. A) Schematic illustration of the experiment design. Human endometrial tissue was inoculated into the peritoneal cavity of mice (n=15; 13 fragments/mouse from one patient) using a micropipette and left for 12 days before starting treatment. On days 12-19 AL8810, Fluprostenol or vehicle (PBS) was injected i.p once a day (n=5/group). B) Representative images captured at time of cull from mice treated with AL8810, Fluprostenol or vehicle. Note presence of endometriotic lesions under bright field (arrows) and human tissue origin confirmed under fluorescence.

Figure 2. A) Histological evaluation of endometrial implants from vehicle, AL8810 and Fluprostenol treated animals. B). Number and size of endometriosis-like lesions as determined at sacrifice in situ; error bars represent mean \pm SD, **, *** $p < 0.01$ and $p < 0.001$, respectively, as compared to the control group. EG = Endometrial Glands, scale bar = 200 μ m.

Figure 3. Real time PCR analysis of the expression of COX1 (A), COX2 (B), AKR-1C3 (C) AKR-1B1 (D), mPGES-1 (E), mPGES-2 (F), cPGES (G) and 15-PGDH (H) in endometriotic lesions. Lesions were harvested from mice treated with vehicle (control), AL8810 or Fluprostenol. mRNA concentrations were normalized to that of the house-keeping gene GAPDH.

Results were from 5 control mice, 5 mice treated with AL8810 and 5 mice treated with Fluprostenol. Data are mean \pm SEM. **, ***, $p < 0.01$ and $p < 0.001$, respectively.

Figure 4. Real time PCR analysis of the expression of MMP9 (A), TIMP1 (B) and VEGF (C) in endometriotic lesions. Lesions were harvested from mice treated with vehicle (control), AL8810 or Fluprostenol. mRNA levels were normalized to that of the house-keeping gene GAPDH.

Results were from 5 control mice, 5 mice treated with AL8810 and 5 mice treated with Fluprostenol. Data are mean \pm SEM. *, **, ***, $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively

Figure 5. Real time PCR analysis of the expression of Bax (A) and Bcl-2 (B) in endometriotic lesions. Lesions were harvested from mice treated with vehicle (control), AL8810 or Fluprostenol. mRNA levels were normalized to that of the house-keeping gene GAPDH. Results were from 5 control mice, 5 mice treated with AL8810 and 5 mice treated with Fluprostenol. Data are mean \pm SEM. *, **, $p < 0.05$ and $p < 0.01$ respectively

Figure 6. Representative immunohistochemical staining of PCNA in endometriotic lesions from Vehicle, AL8810 or Fluprostenol treated mice. Sections from proliferative phase of human endometrium were used as a positive control and the same sections incubated without the primary antibody were used as negative control for immunostaining; scale bar = 20 μ m.

Figure 7. Representative immunohistochemical staining of vWF in endometriotic lesions from, Vehicle, AL8810 or Fluprostenol treated mice. Sections from human endometrium were used as a positive control and the same sections incubated without the primary antibody were used as negative control for immunostaining; scale bar = 20 μ m.

Table 1. List of Primers

Gene	Forward Primer (5'-3')	Reverse Primer(5'-3')
Cox-1	GACCCGCCTCATCCTCATAG	TTGGAACTGGACACCGAACA
Cox-2	TCCCTTGGGTGTCAAAGGTAA	AAAACTGATGCGTGAAGTGCTG
mPGES-1	GGATGCACTTCCTGGTCTTC	TCACGGAGCGGATGGGT
mPGES-2	CTCATCAGCAAGCGACTCAA	CACGCAGCACGCCATA
cPGES-	AGCCTGCTTCTGCAAAGTG	TCCTCCGAGACAACCTGAATG
AKR-1C3	TGTATTGGGATTTGGCACCTA	CAACCTGCTCCTCATTATTGTAT
AKR-1B1	TCGCAGCCAAGCACAAT	CAACAAGGCACAGACCCTC
15-PGDH	AAGCAAAATGGAGGTGAAGG	CCAACTATGCCATGCTTTGA
MMP-9	TTGACAGCGACAAGAAGTGG	CCCTCAGTGAAGCGGTACAT
TIMP-1	GAGAAGGAAGTGGACTCTGGAAAC	AAACTCTATATCCTTCTCAGGCC
VEGF	GCTCTACCTCCACCATGCCA	CACCACTTCGTGATGATTCTGC
Bax	TCAACTGGGGCCGGGTTGTC	CCTGGTCTTGGATCCAGCCCAAC
Bcl-2	GGCACACGCCCCATCCAGCC	GCCGGGGGCAGCCGGGGTCT
GAPDH	CAGGGCTGCTTTTAACTCTGG	TGGGTGGAATCATATTGGAACA

A

Endometrial Biopsy
(Chopped ~ 1mm³)



CFDA-SA
labelling



Tissue
Inoculation



Treatment
(IP injection)



Mice
Euthanized

Day 0

Day 12

Day 19

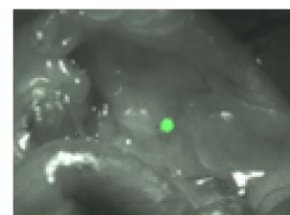
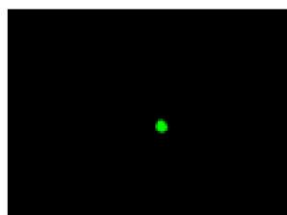
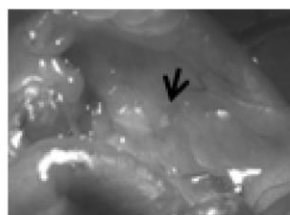
B

Bright Field

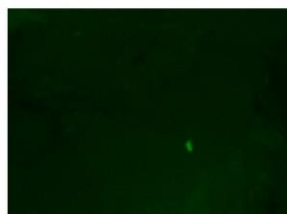
FITC

Merge

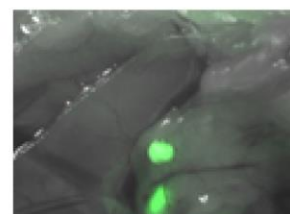
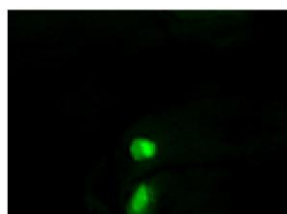
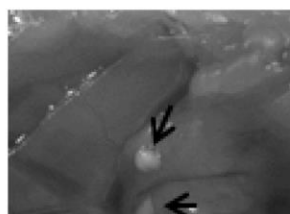
Vehicle



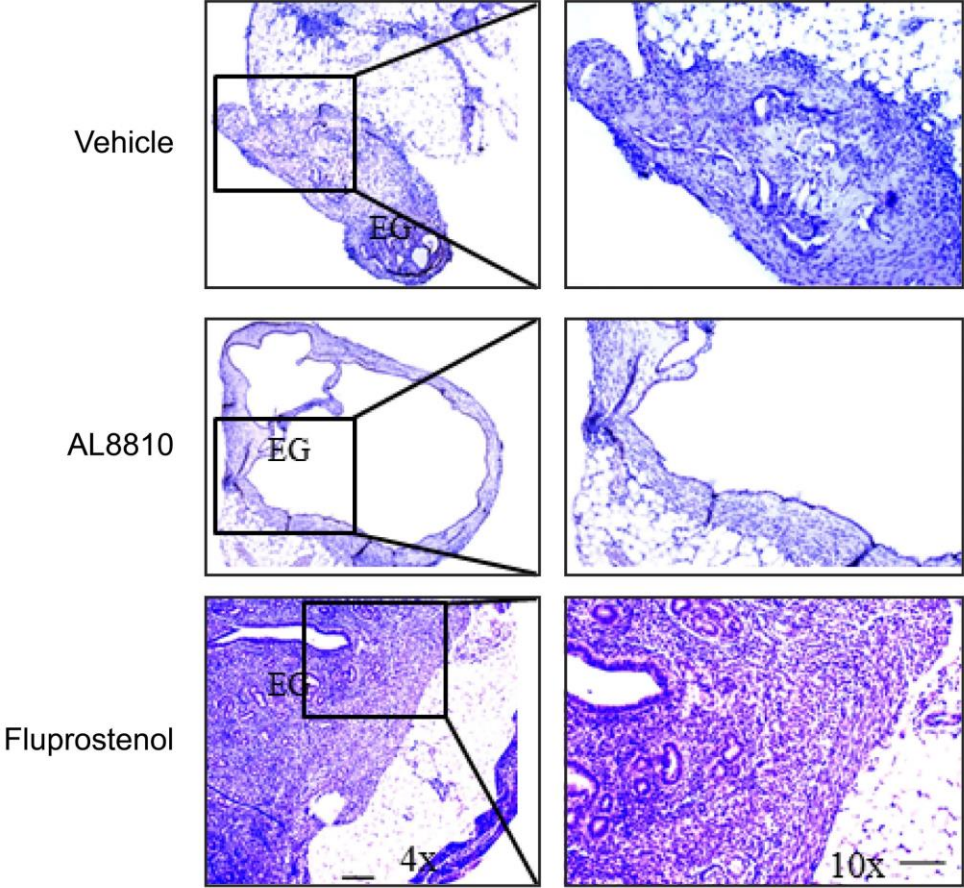
AL8810



Fluprostenol



A



B

